AMERICAN SOCIETY FOR MICROBIOLOGY | Infection and Immunity®

BACTERIAL INFECTIONS

Tracking Anti-*Staphylococcus aureus* Antibodies Produced *In Vivo* and *Ex Vivo* during Foot Salvage Therapy for Diabetic Foot Infections Reveals Prognostic Insights and Evidence of Diversified Humoral Immunity

Irvin Oh,[a] Gowrishankar Muthukrishnan,[a] Mark J. Ninomiya,[a] James D. Brodell, Jr.,[a] Benjamin L. Smith,[a]* Charles C. Lee,[a] Steven R. Gill,[b] Christopher A. Beck,[a,c] Edward M. Schwarz,[a,b] John L. Daiss[a]

[a]Center for Musculoskeletal Research and Department of Orthopaedics, University of Rochester Medical Center, Rochester, New York, USA

[b]Department of Microbiology and Immunology, School of Medicine and Dentistry, University of Rochester, Rochester, New York, USA

[c]Department of Biostatistics and Computational Biology, University of Rochester, Rochester, New York, USA

ABSTRACT Management of foot salvage therapy (FST) for diabetic foot infections (DFI) is challenging due to the absence of reliable diagnostics to identify the etiologic agent and prognostics to justify aggressive treatments. As *Staphylococcus aureus* is the most common pathogen associated with DFI, we aimed to develop a multiplex immunoassay of IgG in serum and medium enriched for newly synthesized anti-*S. aureus* antibodies (MENSA) generated from cultured peripheral blood mononuclear cells of DFI patients undergoing FST. Wound samples were collected from 26 DFI patients to identify the infecting bacterial species via 16S rRNA sequencing. Blood was obtained over 12 weeks of FST to assess anti-*S. aureus* IgG levels in sera and MENSA. The results showed that 17 out of 26 infections were polymicrobial and 12 were positive for *S. aureus*. While antibody titers in serum and MENSA displayed similar diagnostic potentials to detect *S. aureus* infection, MENSA showed a 2-fold-greater signal-to-background ratio. Multivariate analyses revealed increases in predictive power of diagnosing *S. aureus* infections (area under the receiver operating characteristic curve [AUC] $>$ 0.85) only when combining titers against different classes of antigens, suggesting cross-functional antigenic diversity. Anti-*S. aureus* IgG levels in MENSA decreased with successful FST and rose with reinfection. In contrast, IgG levels in serum remained unchanged throughout the 12-week FST. Collectively, these results demonstrate the applicability of serum and MENSA for diagnosis of *S. aureus* DFI with increased power by combining functionally distinct titers. We also found that tracking MENSA has prognostic potential to guide clinical decisions during FST.

KEYWORDS *Staphylococcus aureus*, diabetes, diabetic foot infections, diagnostics, immunoassays, plasmablasts

The growing prevalence of type II diabetes mellitus (DM) in the U.S. and world populations has led to the steadily increasing frequency of its associated sequelae, including diabetic foot infections (DFI) (1, 2). Recent assessments estimate that as many as 26% of Americans over the age of 65 years have type II DM (3, 4). The cost associated with management of DM and its associated ailments is a significant burden on the U.S. health care system (5, 6). In the DM patient population, the risk of developing a foot ulcer is 15%, with two-thirds of lower extremity amputations associated with DFI (1, 7,

Received 12 August 2018 **Returned for modification** 29 August 2018 **Accepted** 20 September 2018

Accepted manuscript posted online 1 October 2018

Citation Oh I, Muthukrishnan G, Ninomiya MJ, Brodell JD, Jr, Smith BL, Lee CC, Gill SR, Beck CA, Schwarz EM, Daiss JL. 2018. Tracking anti-*Staphylococcus aureus* antibodies produced *in vivo* and *ex vivo* during foot salvage therapy for diabetic foot infections reveals prognostic insights and evidence of diversified humoral immunity. Infect Immun 86:e00629-18. https://doi.org/10.1128/IAI.00629-18.

Editor Victor J. Torres, New York University School of Medicine

Address correspondence to John L. Daiss, john_daiss@urmc.rochester.edu.

* Present address: Benjamin L. Smith, Epibone Inc., New York, New York, USA.

I.O. and G.M. contributed equally to this article.

8). Moreover, the 5-year mortality rate of DFI has been reported to be 50%, equal to that of the most life-threatening cancers (9).

Until recently, the primary intervention for DFI was the surgical amputation of the infected part of the foot or lower leg (10, 11). However, recognizing the associated loss of function and an increase in long-term risk, limb salvage treatment has become more prevalent. The limb salvage effort consists of initial surgical debridement of the infected or nonviable part of the foot and obtaining tissue samples for microbial culture to guide a subsequent long-term antibiotic treatment. Typically, a 6-week course of intravenous (i.v.) antibiotics treatment is recommended in the presence of bone infection (12, 13). *Staphylococcus aureus* is the most commonly found pathogen, with a prevalence rate of approximately 50% in patients hospitalized for DFI (14, 15).

The conventional diagnostic method, standard microbiological culture, is vulnerable to sampling error (false positive or false negative), especially in DFI that present with polymicrobial infections. Moreover, differentiating a commensal or bystander pathogen from opportunistic pathogens is challenging. Given the high rate of recurrence that leads to eventual amputation, it is critical for the clinician to monitor treatment response. Continuing an ineffective limb salvage therapy may allow further spread of the infection that requires a more distal amputation from the foot. Therefore, accurate detection of treatment failure is important for a timely surgical intervention to minimize limb loss and optimize physical function. In addition to evaluating the gross changes of the infected wound (size, depth, and wound bed appearance), most clinicians rely on nonspecific inflammatory markers, such as the C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR), for monitoring the treatment response and detecting a recurrent infection. However, these methods are not specific for the pathogen or for the site of infection and are not always definitive measures of treatment success.

To address these central limitations of diagnosis of the etiologic agent in DFI and prognosis of the success of foot salvage therapy (FST), we aimed to develop a blood-based immunoassay that will (i) identify the major infectious pathogen, (ii) effectively monitor treatment response, and (iii) detect recurrence to guide timely intervention. Since our analytic approach is species specific and *S. aureus* is the most common infecting bacterium in DFI, we focused on the presence of antibodies against known *S. aureus* antigens as our initial target. Our approach is based on two fundamental innovations. The first innovation is the measurement of antibodies secreted by recently stimulated, circulating plasmablasts, referred to here as antibody-secreting cells (ASCs) (16–18). The second innovation is a multiplex immunoassay that measures antibodies specific for signature proteins secreted by or expressed on the surface of *S. aureus* (19). We hypothesized that the host response to a combination of antigens will be more powerful in diagnosing *S. aureus* infections than the response to any single antigen.

The introduction of ASCs as a tool for diagnosis and prognosis has been explored in multiple settings but as yet has not been applied in a general medical practice (16–18). From a lymph node draining an infection site, newly stimulated ASCs begin to produce antibody and circulate to other immune organs, such as the spleen and bone marrow (16). Peripheral blood mononuclear cells (PBMCs), which include newly activated ASCs, are harvested from anticoagulated blood, washed extensively in antibody-free medium to remove preexisting serum immunoglobulins, and then cultured *in vitro* in culture medium free of human immunoglobulins to enable constituent ASCs to express antibodies elicited by the ongoing infection. A sample of the resulting medium enriched for newly synthesized antibodies is a potentially important window on the ongoing immune response. In contrast to serum antibodies that remain elevated for months following infection, ASCs emerge into the blood as the ongoing infection develops and decline rapidly as the infections wanes under the attack of the host's immune responses. This attribute makes ASCs potential markers for the initial infection, successful (or failed) therapy, and recurrence. To test this, we performed a proof-of-concept pilot clinical study with 26 DFI patients undergoing FST to demonstrate the

TABLE 1 DFI patient clinical data

Characteristic	Value for:		*P* value
	Patients with *S. aureus* infections	Patients with non-*S. aureus* infections	
% female	25	14	0.6348
Age (yrs)	65 ± 6.6	52 ± 11.6	0.0046
BMI (kg/m^2)	31.6 ± 5.0	33.3 ± 7.3	0.6218
WBC ($\times 10^3/\mu$l)	11,975 ± 6,935.3	10,928.6 ± 3,580.8	0.9296
CRP (mg/dl)	127.7 ± 124.6	94.9 ± 80.9	0.6787
ESR	77.5 ± 60.7	52.2 ± 34.9	0.2384
Diabetes status (A1c level)	9.5 ± 3.1	8.7 ± 2.8	0.6162
Wound healing status (% healed)	58.3	50.0	0.7127

feasibility of assessing medium enriched for newly synthesized anti-*S. aureus* antibodies (MENSA) as a diagnostic for DFI and a prognostic to monitor FST.

RESULTS

Patient demographics and clinical outcome. Twenty-six patients presenting with DFI and eligible for FST were enrolled (Table 1). There were 21 males and 5 females, with an average age of 58 (range, 46.3 to 69.7) years and an average body mass index (BMI) of 32.5 ± 6.3. All patients underwent surgical debridement followed by collection of soft tissue and bone samples for identification of the infecting pathogen. In parallel, wound samples were collected for determination of the most abundant species by 16S rRNA amplicon sequencing. Identification of *S. aureus* by PCR amplification and sequencing of the gene for staphylococcal protein A (*spa*) and/or conventional culture is shown in Table S1 in the supplemental material. Samples of whole blood were collected at the time of enrollment ($t = 0$) for analysis of serum and MENSA as described below. Follow-up samples were collected at 4th, 8th (2 weeks post-FST), and 12th (6 weeks post-FST) weeks.

Among the 26 patients, 3 passed away prior to the end of the 3-month period of examination due to unrelated medical conditions, such as congestive heart failure, and 1 opted for amputation before the end of FST. Overall, 14 had infections in which *S. aureus* was not detectable by culture or *spa* sequencing. Twelve patients with diabetic foot ulcers (DFUs) were infected with *S. aureus*; of these, 10 patients provided at least two samples during the 3-month follow-up period.

Among the 14 DFI patients with no evident colonizing *S. aureus,* the wounds on 8 healed, while the infection in 6 resisted treatment. FST results were similar among the 12 patients in the *S. aureus*-infected cohort: 7 patients healed, while 5 did not. Overall, FST spared more 50% of enrolled subjects from amputation.

Diagnostic potential of anti-*S. aureus* IgG in serum and MENSA. In our initial evaluation of serum and MENSA anti-*S. aureus* IgG as a diagnostic for DFI, we assessd concordance with the patients' clinical PCR results. Consistent with prior reports (19), we found that PCR$^-$ DFI patients had appreciable levels of anti-*S. aureus* antibodies in their sera (Fig. 1A). The immunodominant antigens in this population were the iron-scavenging proteins IsdB and IsdA, along with the autolysin subunits (Amd and Gmd) and the secreted α-toxin (Hla). The same anti-*S. aureus* antibodies predominated among the PCR$^+$ patients but generally at higher levels (Fig. 1A).

In sharp contrast to serum IgG levels, MENSA in *S. aureus* PCR-negative DFI patients were very low (near the limit of detection) for almost all of the antigens (Fig. 1B), suggesting that this approach can overcome the major false-negativity problems associated with serum IgG diagnostics. Additionally, MENSA detected high levels of antibodies against the most immunodominant antigens in the *S. aureus* PCR$^+$ DFI patients (Fig. 1B). By rank ordering the antibody levels, we found that the repertoire of immunodominant antigens in MENSA was similar to that in serum (e.g., IsdA, IsdB, Amd, and Gmd), as well as the increased abundance of antibodies specific for some of the secreted virulence factors, such as staphylococcal inhibitory protein (SCIN) (Fig. 1). To further assess the diagnostic potential of serum and MENSA anti-*S. aureus* IgG, we

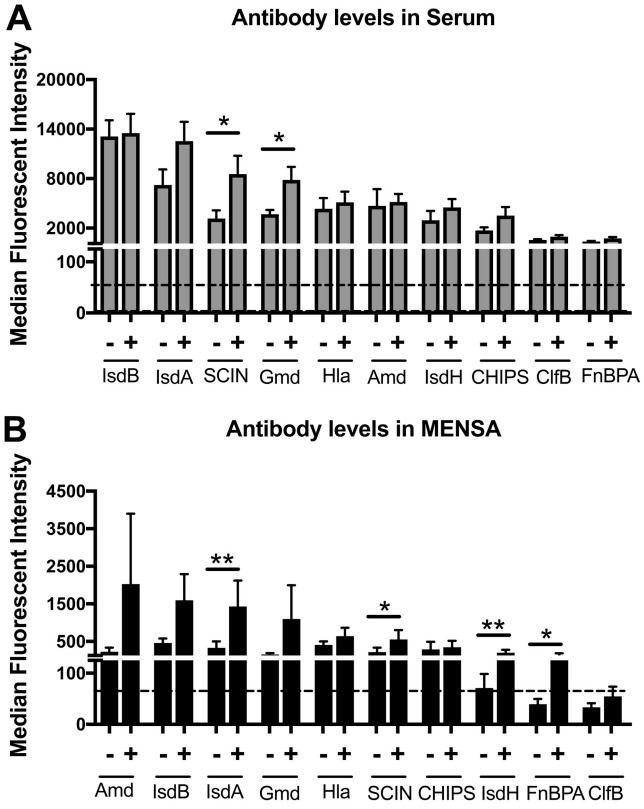

FIG 1 Rank order of immunodominance of the 10 antigens in serum and medium enriched for newly synthesized antibodies (MENSA) from patients with *S. aureus* DFI. (A) The median fluorescent intensity (MFI) ± SEM for each of the 10 antigens was determined via multiplex Luminex immunoassay from serum (diluted 1:10,000) from patients whose diabetic foot ulcers (DFUs) were *spa* PCR negative (−) or positive (+) for *S. aureus* prior to foot salvage therapy. (B) Similarly, anti-*S. aureus* IgG responses were determined from MENSA as described in Materials and Methods. Note that serum antibody and MENSA titers against the iron-sensing determinant (Isd) and autolysin (Amd and Gmd) proteins are greater than those against the immune evasion proteins (Hla, SCIN, and CHIPS) and the adhesins (FnBPA and ClfB). Aggregate lower limit of detection of the assay (calculated by the formula LLOD = assay buffer MFI + 2 × SEM of assay buffer MFI) is depicted by a dashed line. *, $P < 0.05$; **, $P < 0.01$.

determined the ratio of median antibody titers between *S. aureus* PCR^- and PCR^+ DFI patients (Fig. 2). Overall, measurement of anti-*S. aureus* antibodies in both serum and MENSA reliably identified patients with *S. aureus* DFI. However, on average MENSA exhibited a 2-fold-greater signal-to-background ratio.

To formally assess the diagnostic potential of our serum and MENSA immunoassays, we performed receiver operator characteristic (ROC) curve analyses for each antigen (Fig. 3). For serum, IgG antibody titers against Gmd, SCIN, and FnBPA were significantly predictive of *S. aureus* infection (Fig. 3C). Although the results with MENSA were similar to those with serum, IsdH demonstrated remarkable diagnostic potential, as it was the only single antigen with an area under the ROC curve (AUC) of >0.8 tested in either immunoassay (Fig. 3D). MENSA IgGs against IsdA and SCIN were also significantly concordant with PCR detection of *S. aureus* infection in DFI patients.

Combinatorial analysis of antibody response to antigens across functional classes increases diagnostic power for ongoing *S. aureus* infections. In order to improve diagnostic strength of our immunoassays, we performed multivariate analyses on serum and MENSA IgG titers using combinations of antigens chosen either within or across four distinct functional classes (iron acquisition, cell wall metabolism, secreted toxins, and adhesins). The AUCs of the sums of various combinations of antigens are presented in Fig. 4. Interestingly, combining antibody responses against antigens with similar functions failed to increase the diagnostic potential of either the serum or

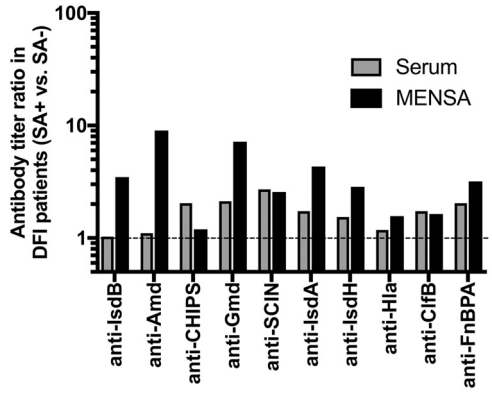

FIG 2 Antigen-specific differential diagnostic potential of serum IgG versus MENSA to detect ongoing *S. aureus* infections in DFU patients. The MFI for each of the 10 antigens studied in this clinical pilot was determined via multiplex Luminex immunoassay from serum (diluted 1:10,000) and MENSA from patients whose DFUs were *spa* PCR negative (SA^-) or positive (SA^+) for *S. aureus* prior to foot salvage therapy. The data are presented as the ratio of median titers in patients with SA^+ DFUs normalized to the median titers in patients with SA^- DFUs. The median background antibody titer level of the SA^- group is represented as a dotted line.

MENSA immunoassay (Fig. 4A and C). In contrast, combination of antibody responses from functionally distinct antigen groups led to significant increases in AUC (Fig. 4B and D). Moreover, only antigen combinations across two or more functional classes yielded AUCs of >0.85. The greatest combination in serum was IsdB-FnBPA-ClfB-SCIN (AUC = 0.93; $P < 0.001$), and the greatest combination in MENSA was Amd-Gmd-IsdA-SCIN (AUC = 0.896; $P < 0.001$). These intriguing results provide the first evidence of cross-functional antigenic biases and suggest that ability to diagnose an ongoing *S. aureus* infection may be improved by increasing antigenic diversity.

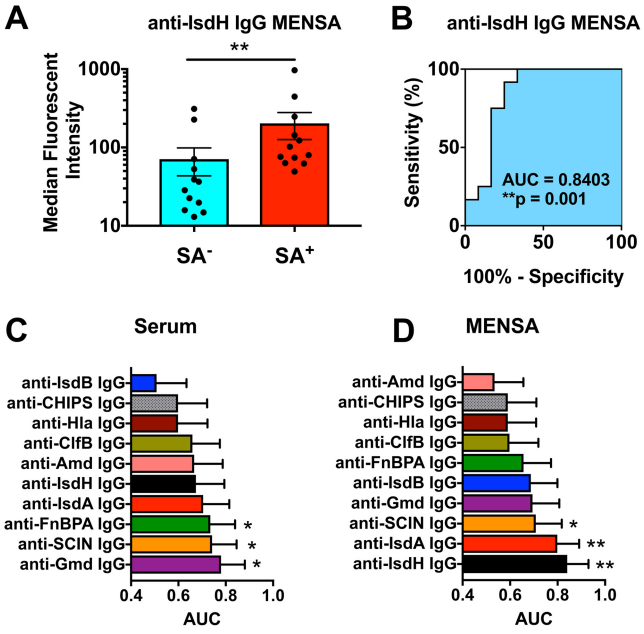

FIG 3 Diagnostic potential of anti-*S. aureus* IgG titers in serum and MENSA for detection of ongoing *S. aureus* infections in DFU patients. The multiplex Luminex data described for Fig. 1 were utilized to generate receiver operating characteristic (ROC) curves for all 10 antigens in serum and MENSA. The primary MENSA data (A) used to generate the area under the curve (AUC) (B) for anti-IsdH IgG are shown to illustrate the single antigen with the greatest diagnostic potential identified in this clinical pilot study. The ROC curves for all 10 antigens are represented in rank order of lowest to highest AUC values in serum (C) and MENSA (D). *, $P < 0.05$; **, $P < 0.01$.

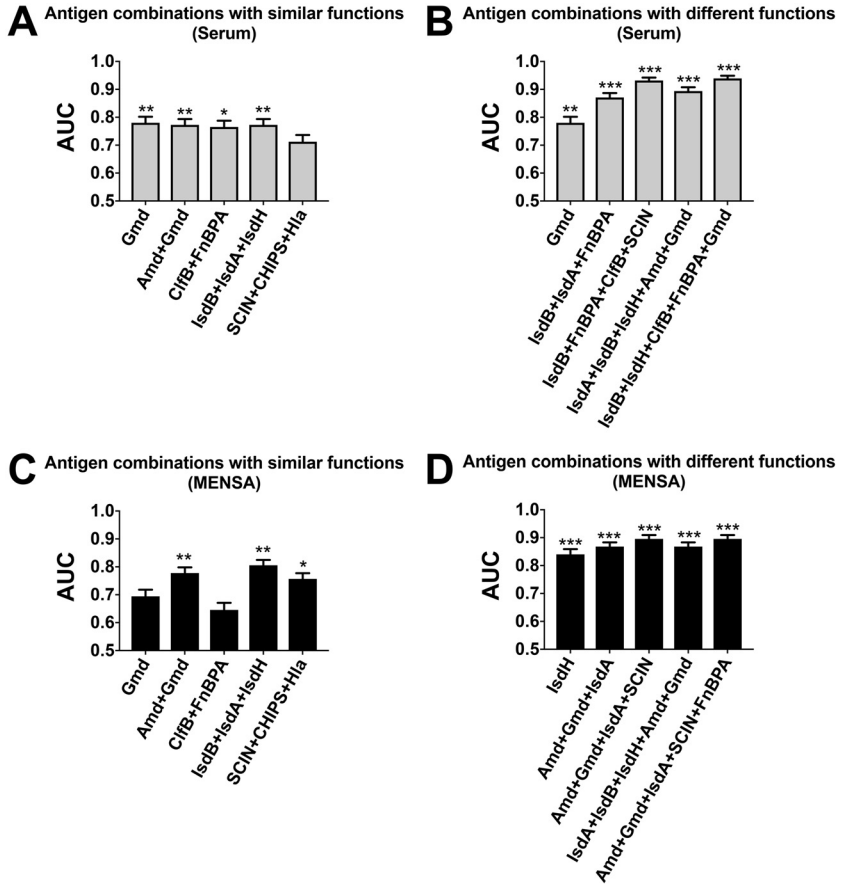

FIG 4 Evidence of diversified humoral immunity in DFU patients with *S. aureus* infections. The AUC values described for Fig. 2 were used to identify combinations of IgG titers in serum (A and B) and MENSA (C and D) with greater diagnostic potential than AUCs of the single antigens. The graphs illustrate results from combinations of antigens with similar (A and C) and different (B and D) functions, and the significance of the AUC value for each antigen or antigen combinations is indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Note that AUC values greater than 0.85 were obtained only by combining functionally distinct antigens.

Anti-*S. aureus* IgG responses in MENSA are superior to serum for tracking treatment response in FST. We also assessed the prognostic potential of our serum and MENSA immunoassays for their ability to track the success or failure of FST. The results showed that serum antibody levels remained unchanged throughout the 3-month course of treatment regardless of clinical outcome (Fig. 5A to C). In contrast, MENSA levels change over time, and their levels correlated with the patient DFI status. As examples, MENSA from patient DFU009, who was PCR$^-$ for *S. aureus,* made essentially no IgG at any time throughout the study period (Fig. 5D). MENSA from patient DFU008, whose *S. aureus* infection healed, contained high levels of antibodies at day 30 that steadily declined throughout the course of FST (Fig. 5E). Finally, patient DFU006, who suffered a recurrent *S. aureus* infection and eventual amputation, demonstrated resurgent high antibody levels in the MENSA (Fig. 5F).

For DFI patients who healed during FST, the difference between an initial high abundance of a specific anti-*S. aureus* antibody and its stable decline may serve as a prognostic measure of recovery. In this pilot study cohort, there were 10 patients who had *S. aureus* infections and yielded enough follow-up samples to track the impact of FST. Six patients achieved complete healing, whereas four suffered either a recurrence or persistent infection. A longitudinal monitoring of anti-Amd IgG levels in healed and nonhealed *S. aureus*-infected DFI patients compared to PCR$^-$ *S. aureus* DFI patients is illustrated in Fig. 6A. Remarkably, the change in anti-Amd levels in MENSA over time

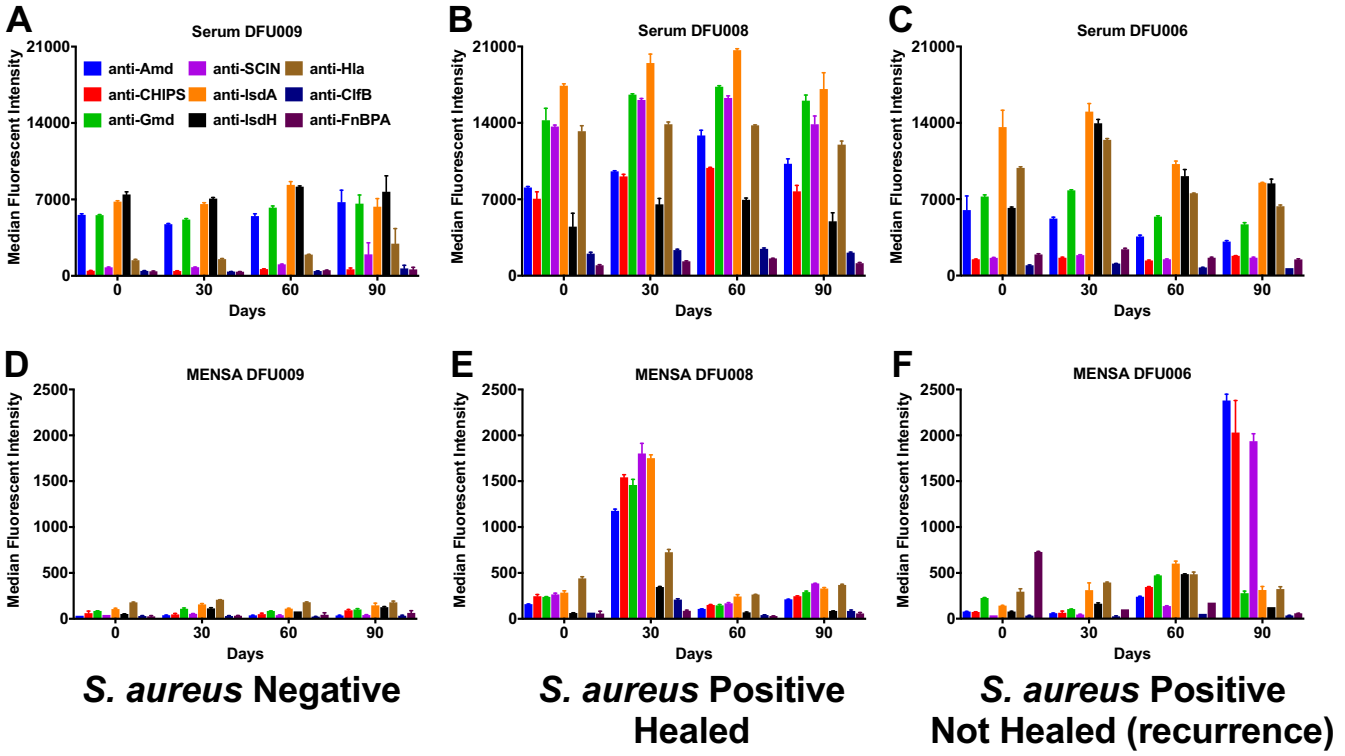

FIG 5 Anti-*S. aureus* IgG responses in MENSA are superior to serum for tracking foot salvage therapy (FST). To assess the change in anti-*S. aureus* antibodies over the course of FST, blood sample collection was performed at the time of enrollment ($t = 0$ weeks) and at 4, 8 and 12 weeks post-FST. Serum (A to C) and MENSA (D to F) IgG levels against the 10 *S. aureus* antigens were determined longitudinally for all patients in the study (MFI ± SEM). Anti-IsdB titers in serum and MENSA remained elevated throughout FST for most patients (data not shown). To illustrate the prognostic potential of serum IgG and MENSA to track FST, longitudinal Luminex multiplex data are presented for a representative patient whose DFU was PCR negative for *S. aureus* (patient DFU009 [A and D]), a patient with *S. aureus*-infected DFU that responded to FST (patient DFU008 [B and E]), and a patient with *S. aureus*-infected DFU for whom FST failed (patient DFU006 [C and F]). Note the absence of remarkable changes in longitudinal serum levels for all three patients. In contrast, MENSA levels faithfully reflected the *S. aureus* infection in these DFU patients over time.

significantly reflected the healing status, with an AUC of 0.875 ($P = 0.006$) (Fig. 6B). Anti-IsdH levels were also significantly predictive of healing, and several other antigen responses demonstrated prognostic trends (Fig. 6C). However, similar monitoring in serum failed to predict a healing response (AUC ~ 0.5).

DISCUSSION

The work presented here demonstrates the potential for two novel analytic approaches: (i) MENSA for tracking rapid changes in the immune response not observable in serum and (ii) multiplex immunoassays to identify infecting pathogens in polymicrobial open-wound infections in DFI patients.

Antibodies as diagnostic markers of infection. Twelve of the 26 patients with DFI were positive for *S. aureus* by either conventional culture or PCR. Each was positive also by the levels of antibodies measured in the serum and specific for characteristic *S. aureus* antigens, notably the iron acquisition proteins IsdH, IsdA, and IsdB and the autolysin proteins Amd and Gmd. We previously demonstrated that antibody responses against IsdA, IsdB, and IsdH could be predictors of infection outcomes in patients with *S. aureus*-induced prosthetic joint infections (PJI). In *S. aureus*-induced PJI, higher anti-IsdA and anti-IsdB antibody levels in patients were associated with morbid outcomes (19). In contrast, anti-Gmd IgG in serum was protective against *S. aureus* infections in patients undergoing orthopedic surgeries (20, 21). Other antigens may also be important. For example, some antibodies specific for certain secretory antigens have been suggested to be earlier signs of infections than antibodies against the cell wall proteins noted here (22, 23).

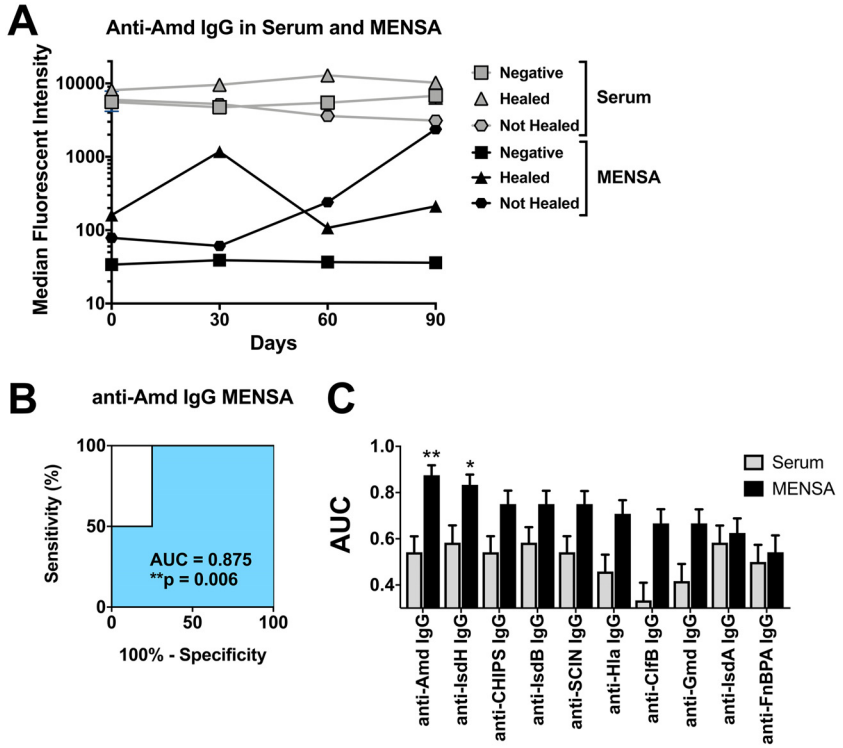

FIG 6 Correlation of anti-*S. aureus* IgG responses in serum and MENSA with healing status during FST. To assess the dynamics of serum and MENSA IgG levels against the 10 antigens over the course of FST, longitudinal Luminex multiplex data for serum and MENSA for all 26 patients in the clinical pilot study were obtained as described for Fig. 5. The prognostic potential of each antigen was assessed by evaluating the anti-IgG levels over the course of FST stratified to three groups: PCR$^-$ for *S. aureus* DFU (negative), *S. aureus*$^+$ DFU responsive to FST (healed), and *S. aureus*$^+$ DFU nonresponsive to FST (not healed). (A) Graph of anti-Amd antibody levels (MFI $\pm$ SEM) over time in serum and MENSA for representative patients—negative (DFU009), healed (DFU008), and not healed (DFU006)—is shown to illustrate the primary results, which were subsequently used to generate ROC curves. Within-patient average change in individual antibody levels for each antigen over time from baseline was calculated and correlated to healing or nonhealing groups among patients with *S. aureus*$^+$ DFU. (B) The ROC curve for anti-Amd IgG in MENSA is shown as an example of an individual antigen (AUC) analysis. (C) Comparison of the ROC curve analyses for all 10 antibodies in serum and MENSA. *, $P < 0.05$; **, $P < 0.01$.

Diagnosis of DFI. Diagnosis of the infecting pathogen in DFI remains a challenge due to the polymicrobial setting of the infection (11, 14, 24). The strength of the blood-borne diagnostic approach is in detection of pathogens that actually cause infection and activate the immune response. Other groups have used oligonucleotide-based microarrays to identify *S. aureus* infections in DFU patients, but DNA-based methods cannot reliably distinguish colonization from infection (25, 26). We found that both serum and MENSA-based assessments of *S. aureus* in a DFI have diagnostic potential. However, our analytical immunoassay using MENSA is superior, as it has higher signal-to-background ratio, making it attractive for clinical diagnostics. Moreover, serum-based testing is likely to suffer from varied background levels resulting from previous infections.

Tracking therapy and measuring recurrence. The greater utility of this proposed analytic strategy lies in its ability to accurately monitor treatment response and to detect persistent or recurrent infection. In patient DFU006, for example, the infected wound healed, at least superficially, but following FST, the persistence or recurrence of *S. aureus* resulted in an infection that required amputation of the patient's limb. While no changes in infection were evident in anti-*S. aureus* antibody levels in the serum, this patient's MENSA response declined at week 8 and dramatically resurged at week 12. In contrast, among the patients who steadily recovered from infection, the antibody response in MENSA became and remained low (e.g., patient DFU008 [Fig. 4B and E]).

Limitations. The current study is preliminary in several ways. First, the study was limited by a small sample size, with only half of the patients presenting with *S. aureus* infections. An increased number of patients will be required to achieve greater statistical power for both the diagnostic and the therapeutic-monitoring applications of this novel analytic approach. Second, a practical diagnostic test will require fewer than the 10 antigens we used in our study. A primary future goal is to identify combinations of the fewest antigens that provide the greatest combined analytic power.

The timing of the ASC response is definitely early in the infection, but it is uncertain where the patient's immune response will be at the time of clinical presentation. Therefore, MENSA-based clinical testing may require a better understanding of the timing of an ongoing infection. Most importantly, true clinical utility for the proposed analytical approach will require the ability to detect and quantitate all bacteria in these polymicrobial infections, including *S. aureus*, *Staphylococcus epidermidis*, *Streptococcus agalactiae*, and *Enterococcus* species. This additional specificity will enable the clinician to selectively target the infecting pathogen in a timely manner, thus improving prognosis.

MATERIALS AND METHODS

Ethics statement. The current study was approved by the University of Rochester Medical Center's Research Subjects Review Board (RSRB; number 00057719). The principal investigator (PI) identified candidate patients. Enrollment, consent procurement, and sampling were performed by PI or clinical coordinators. All personnel involved in the sample collection were institutional review board (IRB)-approved with current Collaborative Institutional Training Initiative (CITI) certification.

Patient enrollment. From July 2015 through the November 2016, we enrolled 26 patients with infected diabetic foot ulcers who displayed clinical symptoms and signs of infection (27) that necessitated hospitalization and undertook FST consisting of surgical irrigation and debridement, followed by a 6-week course of i.v. antibiotic treatment. Patients with both type I and type II DM were included. Patients presenting with a need for immediate major amputation, severe ischemia, venous stasis ulcers, pregnancy, or immune deficiencies and patients who were minors were excluded. DFUs were graded based upon Wagner's classification (28).

All wounds were managed with daily wet-to-dry dressing change until complete healing occurred or amputation was performed. Demographic information, including age, sex, comorbidities, and antibiotics, is summarized in Table 1. The endpoint of the study for each subject was 12 weeks after initiation of FST or the time of definitive amputation as a consequence of treatment failure, whichever occurred first.

Clinical measures to define healing versus nonhealing groups. For each subject, wound size was measured and depth was classified according to Wagner's classification (29). A healing wound is defined as a wound that has completely healed or shows decreased size and depth and an absence of 12 signs and symptoms of infection. A nonhealing wound is defined as a wound that is unimproved or shows increased size and depth with 12 persistent signs and symptoms of infection (29).

Diabetic foot ulcer tissue collection and extraction of DNA. At 0, 4, 8, and 12 weeks after enrollment, wound samples from the patients' infected DFUs were obtained for both standard culture and 16S rRNA sequencing analysis. Tissue specimens were obtained at two different sites for each patient: one at the superficial layer of the wound and the other at the deep tissue level. These specimens were collected aseptically during debridement or as infected tissue during irrigation and debridement surgery. Samples were frozen at −80°C and transferred to the lab. The specimen was then thawed on ice and transferred to FastPrep tubes (MP Biomedicals) that contain 1.4-mm and 0.1-mm spherical silica beads, 750 μl of lysis buffer (Zymo Research), and proteinase K (Sigma). Samples were mechanically lysed with a FastPrep-24 instrument (MP Biomedicals). DNA from the lysate was purified using the ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research).

Microbiological analysis to identify bacterial species. The abundance of bacteria was determined with quantitative real-time PCR (qPCR) using bacterial 16S primers, probe, and cloned plasmid standards. The region of bacterial 16S rRNA from V1 to V3 was amplified from extracted sample DNA using dual-indexed coded primers and Phusion high-fidelity polymerase (Thermo Fisher). All PCR amplicons were purified and normalized using SequalPrep normalization plates (Life Technologies), pooled, and validated on an Agilent bioanalyzer (2200 TapeStation, D1000 tape or HSD1000 tape). The final library was paired-end sequenced (2 × 300 bp) on an Illumina MiSeq. In each amplification and sequencing run, a blank sample was carried through the extraction, amplification, and sequencing steps as a negative control. Samples with bacterial genomic DNA from multiple genera were included as positive controls. In order to further confirm if the infection was *S. aureus* positive, bacterial cultures were further genotyped for the staphylococcal virulence gene *spa*, using primers and PCR conditions as previously described (30, 31); DFI patients were grouped as PCR^+ or PCR^- for *S. aureus* based on this test.

Whole-blood processing. Two tubes (one green-topped sodium heparin tube and one red-topped serum tube) of whole blood were drawn from the study subjects at each time point. The green-topped tube was used immediately for isolating peripheral blood mononuclear cells (PBMCs) using the Ficoll-Paque lymphocyte preparation method. Four to 10 ml of anti-coagulated whole blood from the green-topped tube was diluted with 2 volumes of phosphate-buffered saline (PBS), then layered over 15 to

20 ml of Ficoll-Paque lymphocyte separation medium in a 50-ml conical tube, and centrifuged for 30 min at 1,200 × *g* at room temperature (RT). The resulting PBMC layer was harvested, treated with ammonium chloride-potassium lysing buffer (Gibco) to remove contaminating red blood cells, and then washed extensively in sterile PBS to remove serum immunoglobulins. Total washes reduced the contamination of soluble serum immunoglobulin by at least 10 orders of magnitude. Harvested PBMCs were cultured at 10^6/ml in RPMI 1640 supplemented with antibiotics, glutamine, and 20% fetal bovine serum at 37°C in 5% CO_2 for 72 h. PBMCs were removed by centrifugation, and the resulting MENSA (medium enriched for newly synthesized anti-*S. aureus* antibodies) was harvested and frozen at −80°C in aliquots for subsequent analysis (17, 18). Blood collected in the red-topped tube was allowed to clot, and serum was then collected following centrifugation and stored in aliquots for later analysis. As part of standard clinical care, serum samples collected at each patient visit were tested for erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), white blood count (WBC), HgA1c, and nonfasting glucose level (Table 1). For each consenting patient, whole-blood samples were obtained at baseline ($t = 0$ weeks) and at 4, 8, and 12 weeks.

Multiplex Luminex immunoassay. Anti-*S. aureus* antibody levels in the DFI patients were determined using a custom multiplex Luminex immunoassay developed by our group (19). Briefly, avidin-coated magnetic LumAvidin microspheres with unique spectral signatures were coupled to individual recombinant *S. aureus* antigens as described previously (19). *S. aureus* antigens, from distinct functional classes, were chosen and used for the immunoassay. These include (i) iron acquisition proteins, e.g., iron-regulated surface determinant proteins (IsdA, IsdB, and IsdH), (ii) cell division proteins, e.g., glucosaminidase (Gmd) and amidase (Amd), (iii) immune evasion proteins and secreted toxins, e.g., α-hemolysin (Hla), chemotaxis inhibitory protein of *Staphylococcus* (CHIPS), and staphylococcal inhibitory protein (SCIN), and (iv) cell attachment proteins, e.g., clumping factor B (ClfB) and fibrinogen binding protein A (FnBPA). The design, production, and characterization of these 10 antigens have been described previously (19).

For detecting *S. aureus* antigen-reactive IgG, 1,000 magnetic microspheres per analyte per well were mixed, sonicated, and incubated with 100 μl of diluted serum (1:1,000 or 1:10,000, run in duplicate) or MENSA for 2 h. After a washing, the secondary phycoerythrin-conjugated goat anti-human IgG (Southern Biotech) was added and incubated for 1 h. All serum and MENSA samples were run on a flow cytometer (Bio-Plex 200; Bio-Rad, Life Sciences Research). The fluorescence intensity of the beads and phycoerythrin (100 beads per analyte per well) were acquired for analysis. The data generated from the multiplex immunoassay were accepted for downstream analyses only if the coefficient of variation (CV; ratio of standard deviation to the mean) among the replicates was less than 20%. The aggregate lower limit of detection (LLOD) for all antigens was calculated using the formula LLOD = assay buffer MFI + 2 × SEM of assay buffer MFI, where SEM is standard error of the mean and MFI is median fluorescent intensity.

Statistical data analysis. Patient characteristics were compared for those with versus without infection using the Wilcoxon rank sum test for ordinal and continuous variables and Fisher's exact test for categorical variables. Antibody measurements obtained from serum and MENSA were assessed for predictive ability in discriminating infection status and healing status using receiver operating characteristic (ROC) curve analysis, with overall accuracy summarized by the area under the ROC curve (AUC). Baseline antibody values represented infection status. A longitudinal measure of antibody activity defined as the average change per week from baseline was used to track healing versus nonhealing host responses. ROC curves were analyzed for each individual and combinations of antibodies. Combinations were formed using best-subset selection with multivariate logistic regression models to identify groups of antibodies that best diagnosed and tracked infection versus healing response. The estimated linear predictor from each of these logistic models was converted to a single numerical score for analysis. Nonparametric estimates and 95% confidence intervals for the AUC were computed for each predictor. *P* values for testing the significance of each AUC and for comparing AUCs across predictors were also computed. Analyses were conducted using SAS version 9.4 and R version 3.5.1. A *P* value of less than 0.05 was considered significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00629-18.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

ACKNOWLEDGMENTS

This work was supported by the Goldstein Award, an intradepartmental program in the Department of Orthopaedics for the support of new clinical investigators, awarded to Irvin Oh. This work was also supported by the National Institute of Allergy and Infectious Diseases through grant number R21 AI119646, awarded to John L. Daiss. This work was also supported by National Institute of Arthritis and Musculoskeletal and Skin Diseases through grant number P50AR072000, awarded to Edward M. Schwarz. In addition, this study continued work supported by AOTrauma (Davos, Switzerland) through the AOTrauma Clinical Priority Program on Bone Infection via an award to Stephen Kates.

We thank Ann Gill for expert laboratory assistance, Alex Grier for genomic analysis, and Jamie Colaprete for assistance with sample collection and documentation.

Edward M. Schwarz is a founder of Telephus Medical LLC (San Diego, CA). John L. Daiss is a cofounder of MicroB-plex, Inc. (Atlanta, GA), and works there part-time.

REFERENCES

1. Armstrong DG, Boulton AJM, Bus SA. 2017. Diabetic foot ulcers and their recurrence. N Engl J Med 376:2367–2375. https://doi.org/10.1056/NEJMra1615439.
2. Kwon KT, Armstrong DG. 2018. Microbiology and antimicrobial therapy for diabetic foot infections. Infect Chemother 50:11–20. https://doi.org/10.3947/ic.2018.50.1.11.
3. Centers for Disease Control and Prevention. 2017. National diabetes statistics report, 2017. Centers for Disease Control and Prevention, Atlanta, GA.
4. Gregg EW, Cheng YJ, Srinivasan M, Lin J, Geiss LS, Albright AL, Imperatore G. 2018. Trends in cause-specific mortality among adults with and without diagnosed diabetes in the USA: an epidemiological analysis of linked national survey and vital statistics data. Lancet 391:2430–2440. https://doi.org/10.1016/S0140-6736(18)30314-3.
5. Skrepnek GH, Mills JL, Sr, Armstrong DG. 2015. A diabetic emergency one million feet long: disparities and burdens of illness among diabetic foot ulcer cases within emergency departments in the United States, 2006–2010. PLoS One 10:e0134914. https://doi.org/10.1371/journal.pone.0134914.
6. Hicks CW, Selvarajah S, Mathioudakis N, Sherman RE, Hines KF, Black JH, III, Abularrage CJ. 2016. Burden of infected diabetic foot ulcers on hospital admissions and costs. Ann Vasc Surg 33:149–158. https://doi.org/10.1016/j.avsg.2015.11.025.
7. Neville RF, Kayssi A, Buescher T, Stempel MS. 2016. The diabetic foot. Curr Probl Surg 53:408–437. https://doi.org/10.1067/j.cpsurg.2016.07.003.
8. Boulton AJ, Vileikyte L, Ragnarson-Tennvall G, Apelqvist J. 2005. The global burden of diabetic foot disease. Lancet 366:1719–1724. https://doi.org/10.1016/S0140-6736(05)67698-2.
9. Robbins JM, Strauss G, Aron D, Long J, Kuba J, Kaplan Y. 2008. Mortality rates and diabetic foot ulcers: is it time to communicate mortality risk to patients with diabetic foot ulceration? J Am Podiatr Med Assoc 98:489–493. https://doi.org/10.7547/0980489.
10. Giurato L, Vainieri E, Meloni M, Izzo V, Ruotolo V, Fabiano S, Pampana E, Lipsky B, Gandini R, Uccioli L. 2015. Limb salvage in patients with diabetes is not a temporary solution but a life-changing procedure. Diabetes Care 38:e156–e157. https://doi.org/10.2337/dc15-0989.
11. Lipsky BA. 2015. Stopping antibiotic therapy for a diabetic foot infection: some answers, but more questions. Int J Low Extrem Wounds 14:307–308. https://doi.org/10.1177/1534734615607145.
12. Vuorisalo S, Venermo M, Lepantalo M. 2009. Treatment of diabetic foot ulcers. J Cardiovasc Surg (Torino) 50:275–291.
13. Kim BS, Choi WJ, Baek MK, Kim YS, Lee JW. 2011. Limb salvage in severe diabetic foot infection. Foot Ankle Int 32:31–37. https://doi.org/10.3113/FAI.2011.0031.
14. Lipsky BA. 2004. A report from the international consensus on diagnosing and treating the infected diabetic foot. Diabetes Metab Res Rev 20:S68–S77. https://doi.org/10.1002/dmrr.453.
15. Lipsky BA, Aragon-Sanchez J, Diggle M, Embil J, Kono S, Lavery L, Senneville E, Urbancic-Rovan V, Van Asten S, International Working Group on the Diabetic Foot, Peters EJ. 2016. IWGDF guidance on the diagnosis and management of foot infections in persons with diabetes. Diabetes Metab Res Rev 32:45–74. https://doi.org/10.1002/dmrr.2699.
16. Carter MJ, Mitchell RM, Meyer Sauteur PM, Kelly DF, Truck J. 2017. The antibody-secreting cell response to infection: kinetics and clinical applications. Front Immunol 8:630. https://doi.org/10.3389/fimmu.2017.00630.
17. Lee FE, Falsey AR, Halliley JL, Sanz I, Walsh EE. 2010. Circulating antibody-secreting cells during acute respiratory syncytial virus infection in adults. J Infect Dis 202:1659–1666. https://doi.org/10.1086/657158.
18. Lee FE, Halliley JL, Walsh EE, Moscatiello AP, Kmush BL, Falsey AR, Randall TD, Kaminiski DA, Miller RK, Sanz I. 2011. Circulating human antibody-secreting cells during vaccinations and respiratory viral infections are characterized by high specificity and lack of bystander effect. J Immunol 186:5514–5521. https://doi.org/10.4049/jimmunol.1002932.
19. Nishitani K, Beck CA, Rosenberg AF, Kates SL, Schwarz EM, Daiss JL. 2015. A diagnostic serum antibody test for patients with Staphylococcus aureus osteomyelitis. Clin Orthop Relat Res 473:2735–2749. https://doi.org/10.1007/s11999-015-4354-2.
20. Gedbjerg N, LaRosa R, Hunter JG, Varrone JJ, Kates SL, Schwarz EM, Daiss JL. 2013. Anti-glucosaminidase IgG in sera as a biomarker of host immunity against Staphylococcus aureus in orthopaedic surgery patients. J Bone Joint Surg Am 95:e171. https://doi.org/10.2106/JBJS.L.01654.
21. Varrone JJ, de Mesy Bentley KL, Bello-Irizarry SN, Nishitani K, Mack S, Hunter JG, Kates SL, Daiss JL, Schwarz EM. 2014. Passive immunization with anti-glucosaminidase monoclonal antibodies protects mice from implant-associated osteomyelitis by mediating opsonophagocytosis of Staphylococcus aureus megaclusters. J Orthop Res 32:1389–1396. https://doi.org/10.1002/jor.22672.
22. Verkaik NJ, Boelens HA, de Vogel CP, Tavakol M, Bode LG, Verbrugh HA, van Belkum A, van Wamel WJ. 2010. Heterogeneity of the humoral immune response following Staphylococcus aureus bacteremia. Eur J Clin Microbiol Infect Dis 29:509–518. https://doi.org/10.1007/s10096-010-0888-0.
23. den Reijer PM, Lemmens-den Toom N, Kant S, Snijders SV, Boelens H, Tavakol M, Verkaik NJ, van Belkum A, Verbrugh HA, van Wamel WJ. 2013. Characterization of the humoral immune response during Staphylococcus aureus bacteremia and global gene expression by Staphylococcus aureus in human blood. PLoS One 8:e53391. https://doi.org/10.1371/journal.pone.0053391.
24. Lipsky BA, Berendt AR, Embil J, De Lalla F. 2004. Diagnosing and treating diabetic foot infections. Diabetes Metab Res Rev 20:S56–S64. https://doi.org/10.1002/dmrr.441.
25. Sotto A, Richard JL, Jourdan N, Combescure C, Bouziges N, Lavigne JP, Nimes University Hospital Working Group on the Diabetic Foot (GP30). 2007. Miniaturized oligonucleotide arrays: a new tool for discriminating colonization from infection due to Staphylococcus aureus in diabetic foot ulcers. Diabetes Care 30:2051–2056. https://doi.org/10.2337/dc07-0461.
26. Sotto A, Richard JL, Messad N, Molinari N, Jourdan N, Schuldiner S, Sultan A, Carriere C, Canivet B, Landraud L, Lina G, Lavigne JP, French Study Group on the Diabetic Foot. 2012. Distinguishing colonization from infection with Staphylococcus aureus in diabetic foot ulcers with miniaturized oligonucleotide arrays: a French multicenter study. Diabetes Care 35:617–623. https://doi.org/10.2337/dc11-1352.
27. Lipsky BA, Berendt AR, Cornia PB, Pile JC, Peters EJ, Armstrong DG, Deery HG, Embil JM, Joseph WS, Karchmer AW, Pinzur MS, Senneville E, Infectious Diseases Society of America. 2012. 2012 Infectious Diseases Society of America clinical practice guideline for the diagnosis and treatment of diabetic foot infections. Clin Infect Dis 54:e132–e173. https://doi.org/10.1093/cid/cis346.
28. Tabur S, Eren MA, Celik Y, Dag OF, Sabuncu T, Sayiner ZA, Savas E. 2015. The major predictors of amputation and length of stay in diabetic patients with acute foot ulceration. Wien Klin Wochenschr 127:45–50. https://doi.org/10.1007/s00508-014-0630-5.
29. Gardner SE, Hillis SL, Heilmann K, Segre JA, Grice EA. 2013. The neuropathic diabetic foot ulcer microbiome is associated with clinical factors. Diabetes 62:923–930. https://doi.org/10.2337/db12-0771.
30. Shopsin B, Gomez M, Montgomery SO, Smith DH, Waddington M, Dodge DE, Bost DA, Riehman M, Naidich S, Kreiswirth BN. 1999. Evaluation of protein A gene polymorphic region DNA sequencing for typing of Staphylococcus aureus strains. J Clin Microbiol 37:3556–3563.
31. Harmsen D, Claus H, Witte W, Rothganger J, Claus H, Turnwald D, Vogel U. 2003. Typing of methicillin-resistant Staphylococcus aureus in a university hospital setting by using novel software for spa repeat determination and database management. J Clin Microbiol 41:5442–5448. https://doi.org/10.1128/JCM.41.12.5442-5448.2003.